

Plant PRPP amidotransferase

The present invention relates to the identification of plant PRPP
5 amidotransferase (phosphoribosyl-pyrophosphate amidotransferase,
E.C. 2.4.2.14) as novel target for herbicidal active ingredients.
The present invention furthermore relates to DNA sequences
encoding a polypeptide with PRPP amidotransferase activity.
Moreover, the invention relates to the use of a nucleic acid
10 encoding a protein with PRPP amidotransferase activity which
originates from plants for generating an assay system for
identifying herbicidally active PRPP amidotransferase inhibitors
and to plant PRPP amidotransferase identified using this assay
system. The invention furthermore relates to the use of the
15 nucleic acid SEQ-ID No. 1 or SEQ-ID No. 3 encoding plant PRPP
amidotransferase for the generation of plants with an increased
resistance to PRPP amidotransferase inhibitors and for the
generation of plants with a modified purine nucleotide content.
Moreover, the invention relates to a method of eliminating
20 undesired vegetation, where the plants to be eliminated are
treated with a compound which binds specifically to PRPP
amidotransferase encoded by a DNA sequence SEQ-ID No 1 or a DNA
sequence which hybridizes with this DNA sequence, and inhibits
its function.

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Plants are capable of synthesizing their cell components from
carbon dioxide, water and inorganic salts.

This process is only possible by exploiting biochemical reactions
30 for synthesizing organic substances. Nucleotides are synthesized
de novo in plants. Being components of the nucleic acids, they
are particularly important. Covalently bound, nucleotides
activate carbohydrates for polysaccharide biosynthesis. They
furthermore activate head groups for lipid biosynthesis.
35 Nucleotides are involved in virtually all metabolic pathways.
Nucleoside triphosphates, especially ATP, drive most of the
energy-requiring reactions of the cell. Adenine nucleotides are
additionally also found as components in essential factors such
as coenzyme A and in nicotinamide and flavin coenzymes, which are
40 involved in a large number of cellular reactions. The coupled
hydrolysis of guanosine-5'-triphosphate (GTP) defines a direction
of reaction for various cellular processes such as protein
translation, assembly of microtubuli, vesicular transport, signal
transduction and cell division. Furthermore, nucleotides
45 constitute the starting metabolites for the biosynthesis of

methylxanthines such as caffeine and theobromine in the plant family of the Rubiaceae and Theaceae.

Genes which encode PRPP amidotransferase have been isolated from
5 a variety of organisms.

cDNAs which encode PRPP amidotransferase have been isolated and characterized from various bacterial, animal and vegetable organisms. Plant PRPP amidotransferase cDNAs have been isolated
10 via complementation of *E. coli* purF mutants and via DNA hybridization techniques from *Glycine max*, *Vigna aconitifolia* and from *Arabidopsis thaliana* (Ito et al., *Plant Molecular Biology* 26(1994), 529-533; Kim et al., *The Plant Journal* 7(1995), 77-86). Sequence homology suggests that the encoded enzymes as well as
15 the *E. coli* PRPP amidotransferase contain 4Fe-4S clusters. The plant PRPP amidotransferase amino acid sequences, which in comparison with *E. coli* are extended at the N terminus, show similarity to plastid signal sequences.

20 Several PRPP amidotransferase isoenzymes which are expressed differentially are found in plants. The RNA for *Arabidopsis thaliana* AtATase1, for example, accumulates preferentially in the roots, while the AtATase2 transcripts are found predominantly in young leaves and flowers (Ito et al., *Plant Molecular Biology*
25 26(1994), 529-533). In *Vigna aconitifolia*, a PRPP amidotransferase RNA accumulates mainly in root nodules and is induced in root tissues by L-Glutamine (Kim et al., *The Plant Journal* 7(1995), 77-86).

30 Since plants depend on an effective nucleotide metabolism, it can be assumed that the enzymes which are involved in nucleotide biosynthesis are suitable as target for herbicides. Thus, there have already been described active ingredients which inhibit *de novo* purine biosynthesis in plants. An example which may be
35 mentioned is the natural substance hydanthocidin, which, after phosphorylation *in planta* inhibits adenylosuccinate synthetase (ASS); (Siehl et al., *Plant Physiol.* 110(1996), 753-758).

Inhibitors for enzymes of purine biosynthesis are, moreover, also
40 known for their pharmacological action in animals and microorganisms: folate analogs inhibit, inter alia, the enzyme GAR transformylase and have an antiproliferative, antiinflammatory and immunosuppressant action. Mycophenolic acid (MPA), an IMP dehydrogenase inhibitor in the GMP synthetic
45 pathway, has an antimicrobial, antiviral and immunosuppressant action (Kitchin et al., *Journal of the American Academy of*

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Dermatology 37(1997), 445-449).

Bacterial PRPP amidotransferase can be inhibited for example by glutamine antagonists such as, for example, azaserine,
5 6-diazo-5-oxo-L-norleucine (DON) or
L-2-amino-4-oxo-5-chloropentanoic acid and by mercaptopurine and thioguanosine. Glutamine antagonists are not specific to PRPP amidotransferase and also affect other purine biosynthesis enzymes, such as formylglycinamidine ribotide synthase. The
10 efficacy of glutamine antagonists on plant PRPP amidotransferase is still to be proven.

It is an object of the present invention to provide proof that PRPP amidotransferase in plants is a suitable herbicidal target,
15 to isolate a complete plant cDNA encoding the enzyme PRPP amidotransferase and functionally express it in bacterial or eukaryotic cells, and to produce an efficient and simple PRPP amidotransferase assay system for carrying out inhibitor-enzyme binding studies.

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We have found that this object is achieved by the isolation of genes which encode the plant enzyme PRPP amidotransferase, the generation of PRPP amidotransferase antisense constructs, and the functional expression of PRPP amidotransferase in bacterial or
25 eukaryotic cells.

It is an object of the present invention to isolate full-length cDNAs encoding functional PRPP amidotransferase (E.C.2.4.2.14) from tobacco (*Nicotiana tabacum*).

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A first subject-matter of the present invention is a DNA sequence SEQ-ID NO. 1 or SEQ-ID NO. 3 containing the encoding region of a plant PRPP amidotransferase from tobacco, see Example 1.

35 Another subject-matter of the invention is DNA sequences which are derived from SEQ-ID NO. 1 or SEQ-ID NO. 3 or which hybridize with one of these sequences and which encode a protein which has the biological activity of a PRPP amidotransferase.

40 Tobacco plants of the line *Nicotiana tabacum* cv. Samsun NN which carry a PRPP amidotransferase antisense construct have been characterized in greater detail. The plants show different degrees of retarded growth and bleaching of the leaves. The transgenic lines and the progeny of the 1st and 2nd generation
45 showed a reduced growth in soil. Using Northern hybridization, it was detected that the RNA quantity of PRPP amidotransferase was reduced in plants with reduced growth compared with the wild

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type. Furthermore, measurement of the enzyme activity detected that the amount of PRPP amidotransferase activity was reduced in the transgenic lines compared with wild-type plants, see Example 7. Growth retardation and the reduction in PRPP amidotransferase activity correlate. This clear connection identifies PRPP amidotransferase for the first time unambiguously as suitable target protein for herbicidal active ingredients.

To be able to find efficient inhibitors of plant PRPP amidotransferase, it is necessary to provide suitable assay systems with which inhibitor/enzyme binding studies can be carried out. To this end, for example, the complete cDNA sequence of tobacco PRPP amidotransferase is cloned into an expression vector (pQE, Qiagen) and overexpressed in E. coli, see Example 2.

Alternatively, however, it is possible to express the expression cassette containing a DNA sequence of SEQ-ID No. 1 or SEQ-ID NO. 3 for example in other bacteria, in yeasts, fungi, algae, plant cells, insect cells or mammalian cells, see Example 4.

The PRPP amidotransferase protein which is expressed with the aid of the expression cassette according to the invention is particularly suitable for finding inhibitors which are specific to PRPP amidotransferase.

To this end, for example, the plant PRPP amidotransferase can be employed in an enzyme assay in which the PRPP amidotransferase activity is determined in the presence and absence of the active ingredient to be tested. A comparison of the two activity determinations allows a qualitative and quantitative statement to be made on the inhibitory behavior of the active ingredient to be tested, see Example 3.

The assay system according to the invention allows a multiplicity of chemicals to be tested rapidly and simply for herbicidal properties. Using this method, substances with a potent action can be selected specifically and reproducibly from amongst a large number of substances, in order that further in-depth tests with which the skilled worker is familiar are carried out subsequently with these substances.

The invention furthermore relates to a method of identifying herbicidally active substances which inhibit the PRPP amidotransferase activity in plants, with the following steps:

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- a) the generation of transgenic plants, plant tissues or plant cells which comprise an additional DNA sequence encoding an enzyme with PRPP amidotransferase activity and which are capable of overexpressing an enzymatically active PRPP amidotransferase;
- b) applying a substance to transgenic plants, plant cells, plant tissue or plant parts and to untransformed plants, plant cells, plant tissue or plant parts;
- c) determining the growth or the viability of the transgenic and the untransformed plants, plant cells, plant tissue or plant parts after application of the chemical substance; and
- d) comparing the growth or the viability of the transgenic and the untransformed plants, plant cells, plant tissue or plant parts after applying the chemical substance;

where a suppression of the growth or the viability of the untransformed plants, plant cells, plant tissue or plant parts, but an absence of potent suppression of the growth or viability of the transgenic plants, plant cells, plant tissue or plant parts, confirms that the substance of b) shows herbicidal activity and inhibits the PRPP amidotransferase enzyme activity in plants.

Another subject-matter of the invention is a method of identifying plant PRPP amidotransferase inhibitors with a potentially herbicidal action by cloning the gene of a plant PRPP amidotransferase, overexpressing it in a suitable expression cassette - for example in insect cells - disrupting the cells and employing the cell extract in an assay system for measuring the enzyme activity in the presence of low-molecular-weight chemicals, either directly or after concentration or isolation of the enzyme PRPP amidotransferase.

Another subject-matter of the invention is compounds with a herbicidal action which can be identified with the above-described assay system.

The invention furthermore relates to a method of eliminating undesired vegetation, where the plants to be eliminated are treated with a compound which binds specifically to plant PRPP amidotransferase and inhibits its function.

Herbicidally active PRPP amidotransferase inhibitors can be employed as defoliants, desiccants, haulm killers and, in particular, as herbicides. Weeds in the widest sense are to be understood as meaning all plants which grow in locations where they are undesired. Whether the active ingredients found with the aid of the assay system according to the invention act as total or selective herbicides depends, inter alia, on the quantity applied.

- 10 Herbicidally active PRPP amidotransferase inhibitors can be used, for example, against the following weeds:

Dicotyledonous weeds of the genera:

- Sinapis, Lepidium, Galium, Stellaria, Matricaria, Anthemis,
15 Galinsoga, Chenopodium, Urtica, Senecio, Amaranthus, Portulaca, Xanthium, Convolvulus, Ipomoea, Polygonum, Sesbania, Ambrosia, Cirsium, Carduus, Sonchus, Solanum, Rorippa, Rotala, Lindernia, Lamium, Veronica, Abutilon, Emex, Datura, Viola, Galeopsis, Papaver, Centaurea, Trifolium, Ranunculus, Taraxacum.

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Monocotyledonous weeds of the genera:

- Echinochloa, Setaria, Panicum, Digitaria, Phleum, Poa, Festuca, Eleusine, Brachiaria, Lolium, Bromus, Avena, Cyperus, Sorghum, Agropyron, Cynodon, Monochoria, Fimbristylis, Sagittaria,
25 Eleocharis, Scirpus, Paspalum, Ischaemum, Sphenoclea, Dactyloctenium, Agrostis, Alopecurus, Apera.

- Subject-matter of the invention are also expression cassettes whose sequence encodes a tobacco PRPP amidotransferase or its functional equivalent. The nucleic acid sequence can be, for example, a DNA or a cDNA sequence.
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- In addition, the expression cassettes according to the invention comprise regulatory nucleic acid sequences which govern the expression of the encoding sequence in the host cell. In accordance with a preferred embodiment, an expression cassette according to the invention encompasses upstream, i.e. at the 5' end of the encoding sequence, a promoter, and downstream, i.e. at the 3' end, a polyadenylation signal and, if appropriate, other regulatory elements which are operatively linked to the encoding sequence for the PRPP amidotransferase gene, which sequence lies between the promoter and the polyadenylation signal. Operative linkage is to be understood as meaning the sequential arrangement of promoter, encoding sequence, terminator and, if appropriate, other regulatory elements in such a manner that each of the
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regulatory elements can function as intended when the encoding sequence is expressed.

An expression cassette according to the invention is generated by
5 fusing a suitable promoter with a suitable PRPP amidotransferase
DNA sequence and a polyadenylation signal using customary
recombination and cloning techniques as they are described, for
example, by T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular
Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold
10 Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman and
L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F.M. et
al., Current Protocols in Molecular Biology, Greene Publishing
Assoc. and Wiley-Interscience (1987).

15 Subject-matter of the invention are also functionally equivalent
DNA sequences which encode a PRPP amidotransferase gene and which
show a sequence homology with the DNA sequence SEQ-ID No. 1 or
SEQ-ID No. 3 of 40 to 100%, based on the total length of the DNA
20 sequence.

Preferred subject-matter of the invention are functionally
equivalent DNA sequences which encode a PRPP amidotransferase
gene and which show a sequence homology with the DNA sequence
25 SEQ-ID No. 1 or SEQ-ID No. 3 of 60 to 100%, based on the total
length of the DNA sequence.

Particularly preferred subject-matter of the invention are
functionally equivalent DNA sequences which encode a PRPP
30 amidotransferase gene and which show a sequence homology with the
DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 of 80 to 100%, based on
the total length of the DNA sequence.

Functionally equivalent sequences which encode a PRPP
35 amidotransferase gene are in accordance with the invention those
sequences which retain the desired functions, despite a deviating
nucleotide sequence. Functional equivalents thus encompass
naturally occurring variants of the sequences described herein,
but also artificial nucleotide sequences, for example those which
40 have been obtained by chemical synthesis and which are adapted to
suit the codon usage of a plant.

A functional equivalent is also to be understood as meaning in
particular natural or artificial mutations of an originally
45 isolated sequence which encodes a PRPP amidotransferase and which
continues to show the desired function. Mutations encompass
substitutions, additions, deletions, exchanges or insertions of

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one or more nucleotide residues. Thus, the present invention for example also extends to those nucleotide sequences which are obtained by modifying this nucleotide sequence. The target of such a modification can be, for example, the further delimitation
5 of the encoding sequence contained therein or else, for example, the introduction of further restriction enzyme cleavage sites.

Functionelle equivalents are also those variants whose function is reduced or increased compared with the starting gene or gene
10 fragment.

In addition, the expression cassette according to the invention can also be employed for the transformation of bacteria, cyanobacteria, yeasts, filamentous fungi and algae, with the
15 purpose of producing sufficient amounts of the enzyme PRPP amidotransferase.

Another subject-matter of the invention is a tobacco protein characterized by the amino acid sequence SEQ-ID NO: 2 or SEQ-ID
20 No. 4 or derivatives or parts of this protein with PRPP amidotransferase activity.

Subject-matter of the invention are also plant proteins with PRPP amidotransferase activity with an amino acid sequence homology to
25 the tobacco PRPP amidotransferase with the SEQ-ID NO: 2 or SEQ-ID NO. 4 of 20 - 100% identity.

Preferred are plant proteins with PRPP amidotransferase activity with an amino acid sequence homology to the tobacco PRPP
30 amidotransferase with the sequences SEQ-ID NO: 2 or SEQ-ID NO. 4 of 50 - 100% identity.

Particularly preferred are plant proteins with PRPP amidotransferase activity with an amino acid sequence homology to
35 the tobacco PRPP amidotransferases with the sequences SEQ-ID NO: 2 or SEQ-ID NO. 4 of 80 - 100% identity.

It was another object of the invention to overexpress the PRPP amidotransferase gene in plants in order to generate plants which
40 tolerate PRPP amidotransferase inhibitors.

Overexpression, in a plant, of the gene sequence SEQ-ID NO. 1 or SEQ-ID NO. 3, which encodes a PRPP amidotransferase, results in an increased resistance to PRPP amidotransferase inhibitors. The
45 transgenic plants generated thus are also subject-matter of the invention.

Expressional efficacy of the recombinantly expressed PRPP amidotransferase gene can be determined, for example, in vitro by shoot-meristem propagation or by a germination test. Moreover, the expression of a PRPP amidotransferase gene which has been
5 altered in terms of type and level, and its effects on the resistance to PRPP amidotransferase inhibitors can be tested in greenhouse experiments using test plants.

Subject-matter of the invention are also transgenic plants,
10 transformed with an expression cassette according to the invention containing the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3, which have been made tolerant to PRPP amidotransferase inhibitors by additionally expressing the DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3, and transgenic cells, tissues, parts and
15 propagation material of such plants. Especially preferred in this context are transgenic crop plants such as, for example, barley, wheat, rye, maize, soya, rice, cotton, sugar beet, canola, sunflowers, flax, hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce and the various tree, nut and grapevine
20 species, and also legumes.

A change in the nucleotide content in plants may be useful under various circumstances. For example, nucleotides are added to plant-based baby formulas to achieve a nutrient composition which
25 corresponds to breast milk. Furthermore, an optimized nucleotide content would be helpful when patients are fed by gastric tube. A reduced purine nucleotide content in nutritional plants is relevant for the dietetic diet of patients suffering from gout. Furthermore, nucleotides make and enhance flavors, so that an
30 altered nucleotide content has an effect on the palatability of plants.

Another subject-matter of the invention are thus plants which, following expression of the DNA sequence SEQ-ID No. 1 or SEQ-ID
35 No. 3 in the plant, have a modified purine nucleotide content. It is preferred to increase the content of the purine nucleotides IMP, AMP and/or GMP, or of their di- or trinucleotides ADP, ATP or GDP and GTP.

40 A plant with a modified purine nucleotide content is generated, for example, by expressing, in the plant, an additional DNA sequence SEQ-ID No. 1 or SEQ-ID No. 3 in sense or antisense orientation. A modified purine nucleotide content means that both plants with an increased purine nucleotide content (in the case
45 of sense orientation) and plants with a reduced guanosine nucleotide content (in the case of sense orientation

[cosuppression] or antisense orientation) can be generated.

An increased purine nucleotide content means for the purposes of the present invention for example the artificially acquired
5 ability of an increased purine nucleotide biosynthesis rate by functionally overexpressing the PRPP amidotransferase gene in the plant in comparison with the non-recombinant plant for the duration of at least one plant generation.

- 10 Another subject-matter of the invention is the use of plant PRPP amidotransferase for altering the methylxanthine concentration in plants.

Particularly preferred are sequences which ensure targeting into
15 the apoplast, into plastids, into the vacuole, into the mitochondrion, into the endoplasmatic reticulum (ER), or which, owing to the absence of suitable operative sequences, ensure that the product remains in the compartment where it is formed, in the cytosol, (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423).

20 For example, the plant expression cassette can be introduced into the plant transformation vector pBinAR, see Example 5.

A suitable promoter of the expression cassette according to the
25 invention is, in principle, any promoter which is capable of governing the expression of foreign genes in plants. It is preferred to use, in particular, a plant promoter or a promoter derived from a plant virus. Particularly preferred is the cauliflower mosaic virus CaMV 35S promoter (Franck et al., Cell
30 21(1980), 285-294). This promoter contains different recognition sequences for transcriptional effectors which, in their totality, lead to permanent and constitutive expression of the gene which has been introduced (Benfey et al., EMBO J., 8 (1989), 2195-2202).

35 The expression cassette according to the invention may also comprise a chemically inducible promoter which allows expression of the exogenous PRPP amidotransferase gene in the plant to be governed at a particular point in time. Such promoters which are
40 described in the literature and which can be used are, inter alia, for example the PRP1 promoter (Ward et al., Plant. Mol. Biol. (1993) 22, 361-366), a salicylic acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 388186), a tetracyclin-inducible promoter (Gatz et al., Plant J.
45 (1992) 2, 397-404), an abscisic acid-inducible promoter (EP0335528) or an ethanol- or cyclohexanone-inducible promoter

(WO 93/21334).

Particularly preferred promoters are furthermore those which ensure expression in tissues or parts of the plant in which the biosynthesis of purines or their precursors takes place. Promoters which ensure leaf-specific expression must be mentioned in particular. Promoters which must be mentioned are the potato cytosolic FBPase or the potato ST-LSI promoter (Stockhaus et al., EMBO J., 8 (1989) 2445-245).

10 A foreign protein can be expressed stably in the seeds of transgenic tobacco plants to an extent of 0.67% of the total soluble seed protein with the aid of a seed-specific promoter (Fiedler and Conrad, Bio/Technology 10 (1995), 1090-1094). The expression cassette according to the invention can therefore contain, for example, a seed-specific promoter (preferably the phaseolin promoter, the USP promoter or the LEB4 promoter), the LEB4 signal peptide, the gene to be expressed and an ER retention signal.

20 The inserted nucleotide sequence encoding a PRPP amidotransferase can be produced synthetically or obtained naturally or contain a mixture of synthetic and natural DNA components. In general, synthetic nucleotide sequences are generated with codons which are preferred by plants. These codons which are preferred by plants can be determined from codons with the highest protein frequency expressed in the plant species of the highest interest. When preparing an expression cassette, a variety of DNA fragments may be manipulated in order to obtain a nucleotide sequence which expediently reads in the correct direction and which is equipped with a correct reading frame. Adaptors or linkers can be added to the fragments in order to link the DNA fragments to each other.

Other suitable DNA sequences are artificial DNA sequences as long as they mediate the desired property by increasing the purine nucleotide content in the plant by overexpressing the PRPP amidotransferase gene in crop plants, as described above by way of example. Such artificial DNA sequences can be determined for example by backtranslating of proteins which have PRPP amidotransferase activity and which have been constructed by means of molecular modeling, or they can be determined by in vitro selection. Especially suitable are encoding DNA sequences which have been obtained by backtranslating a polypeptide sequence in accordance with the host-plant-specific codon usage. The specific codon usage can be determined readily by a skilled worker familiar with methods of plant genetics by means of

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computer evaluations of other, known genes of the plant to be transformed.

Other suitable equivalent nucleic acid sequences according to the invention which must be mentioned are sequences which encode fusion proteins, the component of the fusion protein being a plant PRPP amidotransferase polypeptide or a functionally equivalent part thereof. The second part of the fusion protein can be, for example, another polypeptide with enzymatic activity or an antigenic polypeptide sequence, with the aid of which detection of PRPP amidotransferase expression is possible (for example myc-tag or his-tag). However, it is preferably a regulatory protein sequence such as, for example, a signal or transit peptide, which leads the PRPP amidotransferase protein to the desired site of action.

The promoter and terminator regions according to the invention should expediently be provided, in the direction of transcription, with a linker or polylinker containing one or more restriction sites for insertion of this sequence. As a rule, the linker has 1 to 10, in most cases 1 to 8, preferably 2 to 6, restriction sites. In general, the linker within the regulatory regions has a size less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter according to the invention may be native, or homologous, or else foreign, or heterologous, to the host plant. The expression cassette according to the invention comprises, in the 5'-3' direction of transcription, the promoter according to the invention, any sequence and a region for transcriptional termination. Various termination regions can be exchanged for each other as desired.

Manipulations which provide suitable restriction cleavage sites or which eliminate the excess DNA or restriction cleavage sites may also be employed. *In vitro* mutagenesis, primer repair, restriction or ligation may be used in cases where insertions, deletions or substitutions such as, for example, transitions and transversions, are suitable. Complementary ends of the fragments may be provided for ligation in the case of suitable manipulations such as, for example, restriction, chewing-back or filling overhangs for blunt ends.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which correspond essentially to *Agrobacterium tumefaciens* T-DNA polyadenylation signals, in particular those of the gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J., 3 (1984),

835), or functional equivalents.

To transform a host plant with a DNA encoding PRPP
amidotransferase, an expression cassette according to the
5 invention is incorporated, as insertion, into a recombinant
vector whose vector DNA contains additional functional regulatory
signals, for example sequences for replication or integration.
Suitable vectors are described, inter alia, in "Methods in Plant
Molecular Biology and Biotechnology" (CRC Press, Chapters 6/7,
10 71-119).

The transfer of foreign genes into the genome of a plant is
termed transformation. It exploits the above-described methods
for transforming and regenerating plants from plant tissues or
15 plant cells for transient or stable transformation. Suitable
methods are the protoplast transformation by
polyethylene-glycol-induced DNA uptake, the biolistic method
using the gene gun, electroporation, incubation of dry embryos in
DNA-containing solution, microinjection and
20 agrobacterium-mediated gene transfer. The abovementioned methods
are described by, for example, B. Jenes et al., Techniques for
Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and
Utilization, edited by S.D. Kung and R. Wu, Academic Press
(1993), 128-143, and Potrykus Annu. Rev. Plant Physiol. Plant
25 Molec. Biol. 42 (1991), 205-225. The construct to be expressed is
preferably cloned into a vector which is suitable for the
transformation of *Agrobacterium tumefaciens*, for example pBin19
(Bevan et al., Nucl. Acids Res. 12 (1984), 8711).

30 Agrobacteria transformed with an expression cassette according to
the invention can equally be used in a known manner for
transforming plants, in particular crop plants such as cereals,
maize, soya, rice, cotton, sugar beet, canola, sunflowers, flax,
hemp, potatoes, tobacco, tomatoes, oilseed rape, alfalfa, lettuce
35 and the various tree, nut and grapevine species, and also
legumes, for example by bathing wounded leaves or leaf sections
in an agrobacterial suspension and subsequently growing them in
suitable media.

40 The purine biosynthesis site is generally the leaf tissue, so
that leaf-specific expression of the PRPP amidotransferase gene
is meaningful. However, it is obvious that the purine
biosynthesis need not be limited to the leaf tissue, but may also
take place in all other remaining parts of the plant in a
45 tissue-specific fashion, for example in fatty seeds.

In addition, constitutive expression of the exogenous PRPP amidotransferase gene is advantageous. On the other hand, inducible expression may also be desirable.

- 5 Using the recombination and cloning techniques cited above, the expression cassettes according to the invention can be cloned into suitable vectors which allow them to be multiplied, for example in *E. coli*. Suitable cloning vectors are, inter alia, pBR332, pUC series, M13mp series and pACYC184. Especially
10 suitable are binary vectors which are capable of replication both in *E. coli* and in agrobacteria.

Another subject-matter of the invention relates to the use of an expression cassette according to the invention for transforming
15 plants, plant cells, plant tissues or parts of plants. The preferred purpose of the use is to increase the PRPP amidotransferase content in the plant.

Depending on the choice of the promoter, expression may take
20 place specifically in the leaves, in the seeds or in other parts of the plant. Such transgenic plants and their propagation material and their plant cells, tissue or parts are another subject of the present invention.

25 The invention will now be illustrated by the examples which follow, without being limited thereto.

Examples

30 Recombinant methods on which the use examples are based:

General cloning methods

Cloning methods such as restriction cleavages, agarose gel
35 electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking DNA fragments, transformation of *Escherichia coli* cells, growing bacteria and the sequence analysis of recombinant DNA were carried out as described by Sambrook et al. (1989) (Cold Spring
40 Harbor Laboratory Press: ISBN 0-87969-309-6).

Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using an ABI laser
45 fluorescence DNA sequencer, following the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA, 74(1977), 5463-5467). Fragments resulting from a polymerase chain reaction were

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sequenced and checked to avoid polymerase errors in constructs to be expressed.

Analysis of total RNA from plant tissues

- 5 Total RNA from plant tissues was isolated as described by Logemann et al. (Anal. Biochem. 163(1987), 21). For the analysis, in each case 20 µg of RNA were separated in a formaldehyde-containing 1.5% agarose gel and transferred to nylon
10 membranes (Hybond, Amersham). Specific transcripts were detected as described by Amasino (Anal. Biochem. 152(1986), 304). The DNA fragments employed as probe were radiolabeled with a Random Primed DNA Labeling Kit (Roche, Mannheim) and hybridized by standard methods (see Hybond instructions, Amersham).
15 Hybridization signals were visualized by autoradiography with the aid of Kodak X-OMAT AR films.

- Unless otherwise specified, the chemicals used were analytical grade and obtained from Fluka (Neu-Ulm), Merck (Darmstadt), Roth
20 (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Solutions were made with a refined, pyrogen-free water, termed H₂O hereinbelow, from a Milli-Q water refining system (Millipore, Eschborn). Restriction endonucleases, DNA-modifying enzymes and molecular biologic kits were obtained from AGS (Heidelberg),
25 Amersham (Braunschweig), Biometra (Göttingen), Roche (Mannheim), Genomed (Bad Oeynhausen), New England Biolabs (Schwalbach/Taunus), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt), Pharmacia (Freiburg), Qiagen (Hilden) and Stratagene (Heidelberg). Unless otherwise specified, they
30 were used in accordance with the manufacturer's instructions.

- The bacterial strains used hereinbelow (E. coli, XL-1 Blue) were obtained from Stratagene. E. coli AT 2465 was obtained from the coli genetic stock center (Yale University, New Haven). The
35 agrobacterial strain used for transforming plants (Agrobacterium tumefaciens, C58C1 with plasmid pGV2260 or pGV3850kan) was described by Deblaere et al. (Nucl. Acids Res. 13 (1985), 4777). Alternatively, it is also possible to use the agrobacterial strain LBA4404 (Clontech) or other suitable strains. Vectors
40 which can be used for cloning are pUC19 (Yanish-Perron, Gene 33(1985), 103-119), pBluescript SK- (Stratagene), pGEM-T (Promega), pZerO (Invitrogen), pBin19 (Bevan et al., Nucl. Acids Res. 12(1984), 8711-8720) and pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990), 221-230).

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Example 1

Isolation of cDNAs encoding a functional tobacco PRPP amidotransferase.

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To isolate PRPP-amidotransferase-encoding cDNAs from *Nicotiana tabacum*, an *Arabidopsis* PRPP amidotransferase-encoding cDNA clone (AtATase1; Ito et al., Plant Molecular Biology 26(1994), 529-533; GenBank Accession number D28868) was used as template for
10 generating a hybridization probe by means of PCR.

The reaction mixtures contained approx. 1 ng/ μ l template DNA, 0.5 μ M of the oligonucleotide 5'-cgc tct aga act agt gga tc-3' and 5'-tcg agg tcg acg gta tc-3', 200 μ M deoxy nucleotide
15 (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C, 1.5 mM MgCl₂) and 0.02 U/ μ l Taq polymerase (Perkin Elmer).

The amplification conditions were set as follows:

20 Annealing temperature: 50°C, 1 min
Denaturation temperature: 94°C, 1 min
Elongation temperature: 72°C, 2 min
Number of cycles: 30

25 The resulting 1.9 kb fragment was used for a heterologous screening of a *Nicotiana tabacum* var. SR-1 (Stratagene) cDNA library. 3.0×10^5 lambda phages of the cDNA library were plated onto agar plates with *E. coli* XL1-blue as bacterial strain. The phage DNA was transferred to nitrocellulose filters (Gelman
30 Sciences) by means of standard methods (Sambrook et al. (1989), Cold Spring Harbor Laboratory Press ISBN 0-87969-309-6) and fixed on the filters. The hybridization probe used was the above-described PCR fragment, which was radiolabelled with the aid of the "Multiprime DNA labeling systems" (Amersham Buchler)
35 in the presence of α -³²P-dCTP (specific activity 3000 Ci/mmol) following the manufacturer's instructions. The membranes were hybridized after prehybridization at 60°C in 3 x SSPE, 0.1% sodium dodecyl sulfate (w/v), 0.02% polyvinylpyrrolidone (w/v), 0.02% Ficoll 400 (w/v) and 50 mg/ml calf thymus DNA for approx.
40 12 hours. The filters were subsequently washed for 60 minutes in 2 x SSPE, 0.1% sodium dodecyl sulfate (w/v) at 60°C. Positively hybridizing phages were visualized by autoradiography and singled out by means of standard techniques (Sambrook et al. (1989); Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) and
45 transferred into plasmid (Stratagene).

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Following restriction and sequence analysis, two different clones were identified, Ntpur1.1 (clone 7.2) containing the DNA sequence SEQ-ID No. 1 and Ntpur1.2 (clone 9.2) containing the DNA sequence SEQ-ID No. 3, which encode reading frames with homology to *Arabidopsis thaliana* AtATase1. The amino acid sequences of Ntpur1.1 (SEQ-ID No. 2 - length: 573 amino acids) and Ntpur1.2 (SEQ-ID No. 4 - length: 573 amino acids) show 97% identity, see Table 1. The homology with AtATase1 at amino acid level is 81% in the case of Ntpur1.1 and 85% in the case of Ntpur1.2. The continuous reading frames start with nucleotide base 49 (Ntpur1.1) and 25 (Ntpur1.2) respectively, and are translated into polypeptides 573 amino acids in length.

Table 1

15 Amino acid comparison Ntpur1.1 x Ntpur1.2:

	1	MAATVSTASAAATNKSPLSQPLDKPFCSPSQKLLSLSPKTLPKPYRTLVT	50
			:
	1	MAATVSTASAAATNKYPLSQPLDKPFCSLSQKLLSLSPKTHPKPYRTLIT	50
			:
20	51	ASSKNPLNDVVSFKKSADNTLDSYFDDDKPREECGVVGIYGDSEASRLC	100
			:
	51	ASSKNPLNDVISFKKSADNTLDSYFDDDDKPREECGVVGIYGDSEASRLC	100
			:
	101	YLALHALLHRGQEGAGIVAVNDDVLKSITGVGLVSDVFNESKLDQLPGDM	150
			:
25	101	YLALHALQHRGQEGAGIVAVNDDVLKSITGVGLVSDVFNESKLDQLPGDM	150
			:
	151	AIGHVWYSTAGSSMLKNVQPFVANYKFGSVGVAHNGNLVNYKLLRGELEE	200
			:
	151	AIGHVRYSTAGSSMLKNVQPFVASYKFGSVGVAHNGNLVNYKLLRSELEE	200
			:
30	201	NGSIFNTSSDTEVVLHLIAISKARPFLLRIVEACEKIEGAYSMVFVTEDEK	250
			:
	201	NGSIFNTSSDTEVVLHLIAISKARPFLLRIVEACEKIEGAYSMVFVTEDEK	250
			:
	251	LVAVRDPHGFRPLVMGRRSNGAVVFASSETCALDLIEATYEREVNPGEVVV	300
			:
	251	LVAVRDPHGFRPLVMGRRSNGAVVFASSETCALDLIEATYEREVNPGEVVV	300
35			:
	301	VDKDGVHSIYLMPPHEHKSCIFEHIYFALPNSVVFGRSVYESRRAFGEIL	350
			:
	301	VDKDGVQSICLMPHPERKSCIFEHIYFALPNSVVFGRSVYESRRAFGEIL	350
			:
	351	ATEAPVECDVGIAVPDSGIVAALGYAAKAGVPFQOGLIRSHYVGRFTFIEP	400
			:
40	351	ATEAPVECDVVIAPVDSGVVAALGYAAKAGVPFQOGLIRSHYVGRFTFIEP	400
			:
	401	SQKIRDFGVKLKLSVPRALLEGKRVVVVDDSIVRGTTSSKIVRLLKEAGA	450
			:
	401	SQKIRDFGVKLKLSVPRAVLEGKRVVVVDDSIVRGTTSSKIVRLLKEAGA	450
			:
45	451	KEVHMRIASPPIIASCIYGVDTTPSSDELISNRMSVEEIKEFIGSDSLAFL	500
			:
	451	KEVHMRIASPPIIASCIYGVDTTPSSDELISNRMSVEEIKEFIGSDSLAFL	500

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501 PMDSLNLKLLGNDKSKSFCYACFSGNYPVEPTGKVKRIGDFMDDGLSGDMDS 550

|||||

501 PMDSLNLKLLGNDKSKSFCYACFSGNYPVEPTGKVKRIGDFMDDGLSGDMDS 550

551 IDGGWLPGSSRVQKTILNEVRTG 573

|||||

551 IDGGWLPGSSRVQKTILNEVRTS 573

Compared with bacterial and human PRPP amidotransferase sequences, the plant proteins (Ntpur1.1, Ntpur1.2, AtATasel) show an extended N-terminus with a large proportion of basic amino acids (Table 2), which suggests the function of a transit peptide for plastid import (von Heijne et al., Eur. J. Biochem. 180(1989), 535-545).

Table 2

Sequence comparison of Arabidopsis thaliana (AtATasel), Bacillus subtilis (BacSu_purF), Human (pur1_hum) and Nicotiana tabacum (Ntpur1.1), Ntpur1.2) PRPP amidotransferase proteins.

		1				50
20	AtATasel	-----	-----	-----SLN	QTILLTPINL	SLSSPNPSLN
	BacSu_purF	-----	-----	-----	-----	-----
	Ntpur1	LAPHLLFLLS	SFFPPPMAAT	VSTASAAATN	KSPLSQPLDK	PFCSPSQKL.
	Ntpur1-2	-----LS	SFFPPPMAAT	VSTASAAATN	KYPLSQPLDK	PFCSLSQKL.
	pur1_hum	-----	-----	-----	-----	-----
		51				100
25	AtATasel	LHISLS.FLL	PSPLLLLHSS	MESPPTSPLL	BHPKNNSHAP	FDYHNDEDDE
	BacSu_purF	-----	-----	-----	-----	-----MLAEIK
	Ntpur1	..LSLSPKTL	PKPYRTLVT	SSKNPLNDVV	SFKKSADNTL	DSYFDDDED..
	Ntpur1-2	..LSLSPKTH	PKPYRTLIT	SSKNPLNDVI	SFKKSADNTL	DSYFDDDDD..
	pur1_hum	-----	-----	-----	-----	-----MELEEL
		101				150
30	AtATasel	KPREECGVVG	IYGDPE....	..ASRLFYLA	LHALQHRGQE	GAGIVTVSPE
	BacSu_purF	GLNEECGVFG	IWGHEE....	..APQITYYG	LHSLQHRGQE	GAGIVATDGE
	Ntpur1	KPREECGVVG	IYGDSE....	..ASRLCYLA	LHALLHRGQE	GAGIVAVN.D
	Ntpur1-2	KPREECGVVG	IYGDSE....	..ASRLCYLA	LHALQHRGQE	GAGIVAVN.D
	pur1_hum	GIREECGVFG	CIASGEWPTQ	LDVPBVITLG	LVGLQHRGQE	SAGIVTSDGS
35		151				200
	AtATasel	KV..LQTITG	VGLVSEVFNE	SKLDQL.PGE	FAIAHVRYST	AGASMLKNVQ
	BacSu_purF	K...LTAHKG	QGLITEVFQN	GELSKV.KGK	GAIGHVRYAT	AGGGGYENVQ
	Ntpur1	DV..LKSITG	VGLVSDVFNE	SKLDQL.PGD	MAIGHVWYST	AGSSMLKNVQ
	Ntpur1-2	DV..LKSITG	VGLVSDVFNE	SKLDQL.PGD	MAIGHVRYST	AGSSMLKNVQ
40	pur1_hum	SVPTFKSHKG	MGLVNHVFTE	DNLKKLYVSN	LGIGHTRYAT	TGKCELENCQ
		201				250
	AtATasel	PFV.AGYRFG	SIGVAHNGNL	VNYKTLRAML	EENGSIFFNTS	SDTEVVLHLI
	BacSu_purF	PLLFERSQNG	SLALAHNGNL	VNATQLKQQL	ENQGSIFQTS	SDTEVLAHLI
	Ntpur1	PFV.ANYKFG	SVGVAHNGNL	VNYKLLRGEL	EENGSIFFNTS	SDTEVVLHLI
	Ntpur1-2	PFV.ASYKFG	SVGVAHNGNL	VNYKLLRSEL	EENGSIFFNTS	SDTEVVLHLI
45	pur1_hum	PFVVETLH.G	KIAVAHNGEL	VNAARLRKKL	LRHGIGLSTS	SDSEMITQLL
		251				300
	AtATasel	AISKAR....	..PFFMRIID	ACEKLQGAYS	MVFVTEDEKLV	AVRDPYGFRRP

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BacSu_purF KRSGBF.... ..TLKDQIKN SLSMLKGAYA FLIMTETEMI VALDPNGLRP
Ntpur1 AISKAR.... ..PFLLRIVE ACEKIEGAYS MVFVTEDEKLV AVRDPHGFRP
Ntpur1-2 AISKAR.... ..PFLLRIVE ACEKIEGAYS MVFVTEDEKLV AVRDPHGFRP
pur1_hum AYTPPQEQDD TPDWVARIKN LMKEAPTAYS LLIMHRDVIY AVRDPYGNRP
301 350

5
AtATase1 LVMGR.....R SNGAVVFASE TCALDLIEAT YEREVYPGEV
BacSu_purF LSIGM.....M GD.AYVVASE TCAFDVVGAT YLREVEPGEM
Ntpur1 LVMGR.....R SNGAVVFASE TCALDLIEAT YEREVNPGEV
Ntpur1-2 LVMGR.....R SNGAVVFASE TCALDLIEAT YEREVNPGEV
pur1_hum LCIGRLIPVS DINDKEKKTS ETEGWVVSSE SCSFLSIGAR YYREVLPGEI
351 400

10
AtATase1 LVVDKDG VKS QCLMPKFEPK Q...CIFEHI YFSLPNSIVF GRSVYESRHH
BacSu_purF LIINDEGMKS ERFSMNINRS I...CSMEYI YFSRPDSNID GINVHSARKN
Ntpur1 VVVDKDG VKS IYLMPEPEHK S...CIFEHI YFALPNSVVF GRSVYESRRA
Ntpur1-2 VVVDKDG VKS ICLMPHPEPK S...CIFEHI YFALPNSVVF GRSVYESRRA
pur1_hum VEISRHNVTQ LDIISRSEGN PVAFCIFEYV YFARPDMSFE DQMVYTVRYR
401 450

15
AtATase1 FGEILATESP VECDVVIAPV DSGVVAALGY AAKSGVPFQQ GLIRSHYVGR
BacSu_purF LGKMLAQESA VEADVVTGVP DSSISAAIGY AEATGIPYEL GLIKNRYVGR
Ntpur1 FGEILATEAP VECDVGIAPV DSGIVAALGY AAKAGVPFQQ GLIRSHYVGR
Ntpur1-2 FGEILATEAP VECDVVIAPV DSGVVAALGY AAKAGVPFQQ GLIRSHYVGR
pur1_hum CGQQLAIEAP VDADLVSTVP ESATPAALAY AGKCGLPYVE VLCKNRYVGR
451 500

20
AtATase1 TFIEPSQKIR DFGVKLKLSP VRGVLEGKRV VVVDSDIVRG TTSSKIVRLL
BacSu_purF TFIQPSQALR EQGVRMKLSA VRGVLEGKRV VMVDSDIVRG TTSSRIVTML
Ntpur1 TFIEPSQKIR DFGVKLKLSP VRALLEGKRV VVVDSDIVRG TTSSKIVRLL
Ntpur1-2 TFIEPSQKIR DFGVKLKLSP VRVLEGKRV VVVDSDIVRG TTSSKIVRLL
pur1_hum TFIQPNMRLR QLGVAKKFGV LSDNFKGKRI VLVDSDIVRG NTISPIIKLL
501 550

25
AtATase1 REAGAKEVHM RIASPPIVAS CYYGVDTPSS EELISNRLSV EEINEFIGSD
BacSu_purF REAGATEVHV KISSPPIAHP CFYGIDTSTH EELIASSHSV GEIRQEIGAD
Ntpur1 KEAGAKEVHM RIASPPIIAS CYYGVDTPSS DELISNRMSV EEIKEFIGSD
Ntpur1-2 KEAGAKEVHM RIASPPIIAS CYYGVDTPSS DELISNRMSV EEIKEFIGSD
pur1_hum KESGAKEVHI RVASPPIKYP CFMGINIPTR EELIANKPEF DHLAEYLGAN
551 600

30
AtATase1 SLAFLSFDTL KKHL.....GK... .DSK.SFCYA
BacSu_purF TLSFLSVEGL LKGI.....GRKYD .DSNCGQCLA
Ntpur1 SLAFLPMDSL NKLL.....GN... .DSK.SFCYA
Ntpur1-2 SLAFLPMDSL NKLL.....GN... .DSK.SFCYA
pur1_hum SVVYLSVEGL VSSVQEGIKF KKQKEKKHDI MIQENGNGLE CFEKSGHCTA
601 650

35
AtATase1 CFTGDYPVKP TEVKVVRGGG DFIDDGLVGS FENIEAGWVR -----
BacSu_purF CFTGKYPTIE YQDITVLPVK EAVLTK-----
Ntpur1 CFSGNYPVEP TG.KVKR.IG DFMDDGLSGD MDSIDGGWLP GSSRVQKTIL
Ntpur1-2 CFSGNYPVEP TG.KVKR.IG DFMDDGLSGD MDSIDGGWLP GSSRVQKTIL
pur1_hum CLTGKYPVEL EW-----
651

40
AtATase1 -----
BacSu_purF -----
Ntpur1 NEVRTG
Ntpur1-2 NEVRTS
pur1_hum -----

45

```

20

Example 2

Expression of tobacco PRPP amidotransferase in E. coli

- 5 The purpose of expressing Ntpur1.2 in E. coli was to prove that the Ntpur1.2-encoded PRPP amidotransferase enzyme was active. To this end, a 1523 bp fragment was amplified in a PCR with Pfu polymerase using the oligonucleotides Jle336:
5'-ttttgctagcgaactcggtattttgacg-3' and Jle337:
10 5'-aaaaagatctcaggttctaacttcat -3' and Ntpur1.2 DNA as template. The DNA fragment generated encodes a PRPP amidotransferase enzyme with is truncated N-terminally by 86 amino acids and no longer contains the transit peptide to be received. This truncated form of PRPP amidotransferase enzyme starts N-terminally with the
15 amino acids MDSYFDDDD. Using the oligonucleotides, an NheI cleavage site and a BglII cleavage site were inserted via which the fragment generated was ligated into the NheI- and BamHI-cleaved expression vector pET11a (Novagen).
- 20 For expression, the E. coli strain BL21(DE3)LysS (Novagen) was transformed with the construct pETNtpur1.2 which had thus been generated. Following overnight culture, a day culture was inoculated to $OD_{600} = 0.1$ and, after an $OD_{600} = 0.7$ had been reached, induced with 1mM IPTG. A total cell extract was produced
25 by the pressure disruption method ("French press") in 50mM Tris-HCl, pH 7.4; 150mM NaCl. Following SDS polyacrylamide gel electrophoresis, an overexpressed protein of approx. 65 kDa was excised from the gel. To produce antisera, the protein was injected into rabbits (contractor: Eurogentec, Herstal, Belgium).

30

Example 3

Assay system for measuring the activity of plant PRPP amidotransferase activity

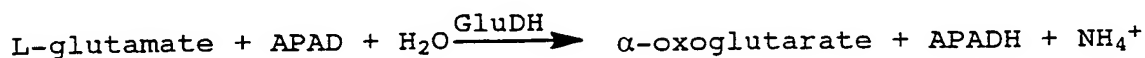
35

- The above-described method for measuring plant PRPP amidotransferase activity by the method of Reynolds et al. (Archives of Biochemistry and Biophysics 229 (1984), 623-631) is not suitable for high-throughput assaying owing to the use of
40 radioactive materials. This is why an alternative assay system with which the plant PRPP amidotransferase activity is detected in the protein extract is detected on the basis of the formation of the reaction product glutamate, based on the method described by Shid and Ishii (Journal of Biological Chemistry 66 (1969),
45 175-181) for E. coli PRPP amidotransferase. The concentration of

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the glutamate which forms is measured by converting it with glutamate dehydrogenase (GluDH) and monitoring APADH formation photometrically at 363 nm.



10 (PRPP = phosphoribosyl pyrophosphate, PRA = phosphoribosylamine, APAD = 3-acetylpyridineadenin dinucleotide, PRAT = PRPP amidotransferase)

To this end, the reaction batch (see below) is incubated at 37°C
15 for up to 60 minutes and the reaction was quenched by incubation at 95°C for 5 minutes.

Reaction batch:

20	375 μl	100 mM	Tris/HCl buffer pH 8.0
	75 μl	100 mM	MgCl ₂
	75 μl	30 mM	phosphoribosyl pyrophosphate
	75 μl	100 mM	L-glutamine
	50 μl		H ₂ O
25	<u>100 μl</u>		protein extract
	750 μl		

The glutamate formed was detected in the detection batch (see below) by measuring the increase in APADH photometrically at
30 363 nm following addition of glutamate dehydrogenase.

Detection batch:

	375 μl	100 mM	Tris/HCl buffer pH 8.0
35	75 μl	500 mM	KCl
	125 μl		H ₂ O
	75 μl	3 mM	APAD
	<u>100 μl</u>		of the reaction batch
	750 μl		

40

Start of the detection reaction with 2 μl (approx. 4 units) glutamate dehydrogenase (Sigma).

The assay system lends itself in particular for measuring PRPP
45 amidotransferase activity from plant material and in expression extracts, for example from baculovirus-infected insect cells.

22

Example 4

Functional expression of tobacco PRPP amidotransferase in insect cells

5

The back-to-back expression system from GibcoBRL was employed for expressing Ntpur1.1 in baculovirus-infected insect cells. To this end, Ntpur1.1 was employed in a PCR. The reaction mixtures contained approximately 1 ng/ μ l Ntpur1.1 DNA, 0.5 μ M of the
10 oligonucleotides 5'-tat agg atc cat gga ctc cta ttt tga cg-3' and 5'-atg aat tct agc tgg ttc taa ctt c-3', 200 μ M deoxynucleotides (Pharmacia), 0.04 U/ μ l Pfu polymerase (Stratagene) and buffer conditions were set following the manufacturer's instructions.

15 The amplification conditions were set as follows:

Step 1:

Denaturation temperature: 95°C, 0.5 min
20 Annealing temperature: 40°C, 0.5 min
Elongation temperature: 72°C, 2 min
Number of cycles for Step 1: 2

Step 2:

25

Denaturation temperature: 95°C, 0.5 min
Annealing temperature: 50°C, 0.5 min
Elongation temperature: 72°C, 3 min
Number of cycles for Step 2: 25

30

The PCR product was ligated into the StuI-cut vector pFastBac1 (GibcoBRL). The correct orientation of the insert was ensured by control digest with KpnI. The resulting transfer vector pFastBacNtpur1.2 was used following the manufacturer's
35 instructions for generating recombinant baculoviruses by means of Sf21 insect cells (Invitrogen). Sf21 insect cells were infected with the recombinant baculovirus (BvNtpur1.2). After 2-4 days, the cells were harvested by centrifugation. A protein of approx. 54kDa, which corresponds to the expected size of PRPP
40 amidotransferase, was identified in the total extract by SDS polyacrylamide gel electrophoresis. A total cell extract was prepared by the pressure disruption method ("French press") in extraction buffer (100 mM HEPES pH 8.0; 2.5 mM EDTA; 10% glycerol; 20 mM DTE; 0.2 mM PEFA block) and, after being freed
45 from salt over a PD10 column (Pharmacia), used for measuring PRPP amidotransferase activity in the assay described (see Example 3).

23

Example 5

Generation of plant transformation vectors

- 5 To generate binary vectors for plant transformation, clone Ntpur1.1 was cleaved with SmaI and EcoRV, and a fragment comprising 1482 bp was isolated and ligated into the SmaI-cleaved vector pBinAR (Höfgen and Willmitzer, Plant Science 66(1990), 221-230). The antisense and sense constructs thus obtained were
10 termed pBinAR-Ntpur1A and pBinAR-Ntpur1, respectively; see Figure 1.

Example 6

15 Generation of transgenic tobacco plants

- Plasmid pBinAR-Ntpur1A and pBinAR-Ntpur1 were transformed into Agrobacterium tumefaciens C58C1:pgV2260 (Deblaere et al., Nucl. Acids. Res. 13(1984), 4777-4788). To transform tobacco plants
20 (Nicotiana tabacum cv. Samsun NN), a 1:50 dilution of an overnight culture of a positively transformed agrobacterial colony in Murashige-Skoog medium (Murashige and Skoog, Physiol. Plant. 15(1962), 473) supplemented with 2% sucrose (2MS medium) was used. Leaf disks of sterile plants (in each case approx.
25 1 cm²) were incubated in a Petri dish for 5-10 minutes with a 1:50 agrobacterial dilution. This was followed by 2 days' incubation in the dark at 25°C on 2MS medium with 0.8% Bacto agar. Cultivation was continued after 2 days and at 16 hours
30 light/8 hours dark and continued in a weekly rhythm on MS medium with 500 mg/l claforan (cefotaxime-sodium), 50 mg/l kanamycin, 1 mg/l benzylaminopurine (BAP), 0.2 mg/l naphthylacetic acid and 1.6 g/l glucose. Growing shoots were transferred to MS medium supplemented with 2% sucrose, 250 mg/l claforan and 0.8% Bacto agar.
- 35 Regenerated shoots were obtained on 2MS medium with kanamycin and claforan, transferred into soil after rooting, and, after cultivation for two weeks in a controlled-environment cabinet in a 16-hour light/8-hour dark rhythm at 60% atmospheric humidity,
40 analyzed for PRPP amidotransferase expression and activity and for altered metabolite contents and phenotypic growth characteristics. Altered nucleotide contents can be determined for example following the method of von Stitt et al., FEBS Letters 145(1982), 217-222.

24

Example 7

Analysis of transgenic plants

5 Transgenic plants which were transformed with the construct with
pBinAR-Ntpurl are characterized by a growth which is reduced by
different degrees and by large-scale bleaching of the leaves in
comparison with untransformed control plants (Fig. 2). RNA
analysis by the Northern blot technique showed a reduced amount
10 of Ntpurl.1-RNA in transgenic lines with the above-described
phenotype (Fig. 3). These effects were also observed in
subsequent generations of the transgenic lines.

To test the correlation with growth reduction, PRPP
15 amidotransferase activity in the transgenic lines was measured
and compared with that in untransformed controls. To this end, in
each case approx. 30 g of leaves from plants approximately 20 cm
in height were homogenized with 50 ml of extraction buffer at
+4°C.

20

Extraction buffer:

100 mM	HEPES pH 8,0
2.5 mM	EDTA
25 10%	glycerol
20 mM	DTE
0,2 mM	PEFA block (40mM)

The disruption extract was filtered through Miracloth
30 (Calbiochem, Bad Soden) and spun at 16,000 rpm in a Sorval
centrifuge. The resulting supernatant was precipitated with
ammonium sulfate at 4°C. The 30% - 60% fraction was solubilized in
an extraction buffer and freed from salt by means of a PD-10
column (Pharmacia, Sweden). The extract thus obtained is stable
35 for at least 24 hours and can be stored over a prolonged period
at -20°C after addition of glycerol (end concentration 50%). The
extract can be employed directly in the activity determination.
Compared to wild-type plants, the PRPP amidotransferase activity
in the transgenic lines was markedly reduced, see Fig. 4. Fig. 4A
40 shows the PRPP amidotransferase activity based on the protein
quantity. Fig. 4B shows the PRPP amidotransferase activity based
on the fresh weight.

25

These data establish a direct connection between reduced PRPP amidotransferase activity and reduced growth of the tobacco plants and thus identify PRPP amidotransferase for the first time as suitable target protein for herbicidal active ingredients.

5

Example 8

Search for PRPP amidotransferase activity inhibitors

- 10 The in-vitro assay described in Example 3 can be used together with high-throughput methods for searching for PRPP amidotransferase activity inhibitors. To this end, the PRPP amidotransferase activity can be prepared from plant tissue, see Example 7. Alternatively, a plant PRPP amidotransferase can be
- 15 expressed in E. coli, insect cells or in another suitable expression system. Known PRPP amidotransferase inhibitors such as glutamine antagonists were identified in this manner.

Example 9

20

Analysis of the adenine and guanine nucleotide contents in transgenic plants.

- Leaf material (in each case 5 disks of 6 mm diameter) was
- 25 harvested from wild-type plants and transgenic plants transformed with the construct pBinAR-Ntpur1 and the subsequent generation (lines 3.1, 3.2, 3.9, 25.1 and 38.8) and frozen immediately in liquid nitrogen. TCA extracts were subsequently prepared by standard methods and employed for the determination of the
- 30 nucleotide contents.

- In the transgenic plants, with the exception of line 38.8, AMP is reduced greatly in the green regions of the leaf and less in the yellow regions of the leaf compared to the wild type (WT) (see
- 35 Fig. 5).

No changes compared to the wild type were observed for the guanosine nucleotide GTP, GDP and GMP.

40

45

SEQUENCE LISTING

<110> BASF Aktiengesellschaft

<120> Plant PRPP amidotransferase

<130> NAE991125

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<170> PatentIn Vers. 2.0

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<212> DNA

<213> Nicotiana tabacum

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<221> CDS

<222> (49)..(1767)

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                                     Met Ala Ala
                                     1

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Thr Val Ser Thr Ala Ser Ala Ala Ala Thr Asn Lys Ser Pro Leu Ser
      5              10              15

cag ccc ctc gac aaa ccc ttt tgc tcc cca tct caa aag ctc tta tct 153
Gln Pro Leu Asp Lys Pro Phe Cys Ser Pro Ser Gln Lys Leu Leu Ser
      20              25              30              35

tta tcc cct aaa acc ctc cca aaa ccc tat aga act ctc gtc acc gca 201
Leu Ser Pro Lys Thr Leu Pro Lys Pro Tyr Arg Thr Leu Val Thr Ala
              40              45              50

tct tcc aaa aac ccc tta aac gac gtc gtt tcg ttt aag aaa tca gct 249
Ser Ser Lys Asn Pro Leu Asn Asp Val Val Ser Phe Lys Lys Ser Ala
              55              60              65

gac aat aca ttg gac tcg tat ttt gac gat gaa gac aaa ccc cgt gaa 297
Asp Asn Thr Leu Asp Ser Tyr Phe Asp Asp Glu Asp Lys Pro Arg Glu
      70              75              80

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gag tgt ggc gtt gtg ggc atc tat ggc gac tca gaa gct tca cgc ctt 345
 Glu Cys Gly Val Val Gly Ile Tyr Gly Asp Ser Glu Ala Ser Arg Leu
 85 90 95

tgc tat tta gca ctt cac gcg ctt cta cac cgt ggc caa gaa ggc gcc 393
 Cys Tyr Leu Ala Leu His Ala Leu Leu His Arg Gly Gln Glu Gly Ala
 100 105 110 115

ggc att gtc gcc gtt aac gac gac gtt ctt aag tca att aca ggt gtt 441
 Gly Ile Val Ala Val Asn Asp Asp Val Leu Lys Ser Ile Thr Gly Val
 120 125 130

ggg tta gta tcc gac gtg ttc aat gag tca aag ctt gac caa ctc cct 489
 Gly Leu Val Ser Asp Val Phe Asn Glu Ser Lys Leu Asp Gln Leu Pro
 135 140 145

ggt gac atg gca att ggc cac gtc tgg tac tct act gct ggc tct tct 537
 Gly Asp Met Ala Ile Gly His Val Trp Tyr Ser Thr Ala Gly Ser Ser
 150 155 160

atg tta aaa aat gtt cag cct ttt gtt gct aat tat aaa ttt ggg tca 585
 Met Leu Lys Asn Val Gln Pro Phe Val Ala Asn Tyr Lys Phe Gly Ser
 165 170 175

gtt ggt gtt gcc cat aat ggt aat tta gtg aat tat aag tta ctg cgt 633
 Val Gly Val Ala His Asn Gly Asn Leu Val Asn Tyr Lys Leu Leu Arg
 180 185 190 195

ggt gaa cta gaa gag aat ggg tca att ttt aat acg agt tct gat act 681
 Gly Glu Leu Glu Glu Asn Gly Ser Ile Phe Asn Thr Ser Ser Asp Thr
 200 205 210

gaa gtg gta ctt cac ctt att gct ata tcg aaa gct agg cct ttt tta 729
 Glu Val Val Leu His Leu Ile Ala Ile Ser Lys Ala Arg Pro Phe Leu
 215 220 225

ttg agg att gtt gag gct tgt gaa aaa att gaa ggt gct tat tct atg 777
 Leu Arg Ile Val Glu Ala Cys Glu Lys Ile Glu Gly Ala Tyr Ser Met
 230 235 240

gtg ttt gtt act gag gat aag ttg gtt gcc gta agg gat cct cat ggg 825
 Val Phe Val Thr Glu Asp Lys Leu Val Ala Val Arg Asp Pro His Gly
 245 250 255

ttt agg cca ttg gtt atg ggt agg aga agt aat ggt gct gtt gtt ttt 873
 Phe Arg Pro Leu Val Met Gly Arg Arg Ser Asn Gly Ala Val Val Phe
 260 265 270 275

3

gcg tcg gag acg tgt gct ttg gat ttg att gag gct act tat gag agg	921
Ala Ser Glu Thr Cys Ala Leu Asp Leu Ile Glu Ala Thr Tyr Glu Arg	
280 285 290	
gag gtg aat cct ggt gag gtt gtt gtt gtg gat aaa gat ggg gtc cat	969
Glu Val Asn Pro Gly Glu Val Val Val Val Asp Lys Asp Gly Val His	
295 300 305	
tct att tat ttg atg cct cat ccc gag cat aaa tct tgt atc ttt gag	1017
Ser Ile Tyr Leu Met Pro His Pro Glu His Lys Ser Cys Ile Phe Glu	
310 315 320	
cat att tac ttt gct ctg cct aat tcg gtc gtg ttt ggg agg tct gtg	1065
His Ile Tyr Phe Ala Leu Pro Asn Ser Val Val Phe Gly Arg Ser Val	
325 330 335	
tac gag tct agg cgt gct ttt gga gag att ctt gcg act gaa gct ccc	1113
Tyr Glu Ser Arg Arg Ala Phe Gly Glu Ile Leu Ala Thr Glu Ala Pro	
340 345 350 355	
gta gaa tgt gat gtt ggg ata gca gtt cct gat tcg ggt atc gtg gct	1161
Val Glu Cys Asp Val Gly Ile Ala Val Pro Asp Ser Gly Ile Val Ala	
360 365 370	
gcg ctc ggt tat gct gct aaa gcg ggg gta ccg ttt caa caa ggt ttg	1209
Ala Leu Gly Tyr Ala Ala Lys Ala Gly Val Pro Phe Gln Gln Gly Leu	
375 380 385	
ata agg tcg cat tat gtt ggt agg aca ttt atc gag ccg tcg cag aag	1257
Ile Arg Ser His Tyr Val Gly Arg Thr Phe Ile Glu Pro Ser Gln Lys	
390 395 400	
ata agg gat ttc ggg gtg aag ctt aag ttg tca cca gtt agg gca tta	1305
Ile Arg Asp Phe Gly Val Lys Leu Lys Leu Ser Pro Val Arg Ala Leu	
405 410 415	
ttg gag ggg aaa agg gtt gtg gtc gtg gac gat tca atc gtt aga ggg	1353
Leu Glu Gly Lys Arg Val Val Val Val Asp Asp Ser Ile Val Arg Gly	
420 425 430 435	
acg acc tcg tcc aag att gtg agg ttg ttg aag gag gcg ggt gcg aaa	1401
Thr Thr Ser Ser Lys Ile Val Arg Leu Leu Lys Glu Ala Gly Ala Lys	
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gag gtt cat atg agg att gca agc cca cca att ata gct tct tgt tat	1449
Glu Val His Met Arg Ile Ala Ser Pro Pro Ile Ile Ala Ser Cys Tyr	
455 460 465	

4

tat gga gtg gat act cct agt tca gat gag ctg ata tca aat agg atg 1497
 Tyr Gly Val Asp Thr Pro Ser Ser Asp Glu Leu Ile Ser Asn Arg Met
 470 475 480

agt gtg gag gag att aag gag ttc att gga tcg gat tcg ctt gct ttt 1545
 Ser Val Glu Glu Ile Lys Glu Phe Ile Gly Ser Asp Ser Leu Ala Phe
 485 490 495

ctg cca atg gat agc ttg aat aag ttg tta ggc aat gat tct aaa agc 1593
 Leu Pro Met Asp Ser Leu Asn Lys Leu Leu Gly Asn Asp Ser Lys Ser
 500 505 510 515

ttt tgc tat gct tgc ttt tcg ggc aat tac ccg gtc gag ccg acg ggt 1641
 Phe Cys Tyr Ala Cys Phe Ser Gly Asn Tyr Pro Val Glu Pro Thr Gly
 520 525 530

aag gtt aaa agg att ggg gat ttc atg gat gat gga tta agt gga gat 1689
 Lys Val Lys Arg Ile Gly Asp Phe Met Asp Asp Gly Leu Ser Gly Asp
 535 540 545

atg gat tcc att gat ggt ggt tgg cta cca gga agt agt agg gtt caa 1737
 Met Asp Ser Ile Asp Gly Gly Trp Leu Pro Gly Ser Ser Arg Val Gln
 550 555 560

aag act atc ttg aat gaa gtt aga acc ggc taaactttct tttccatggt 1787
 Lys Thr Ile Leu Asn Glu Val Arg Thr Gly
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<212> PRT

<213> Nicotiana tabacum

<400> 2

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Pro Leu Ser Gln Pro Leu Asp Lys Pro Phe Cys Ser Pro Ser Gln Lys
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Leu Leu Ser Leu Ser Pro Lys Thr Leu Pro Lys Pro Tyr Arg Thr Leu
 35 40 45

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Val	Thr	Ala	Ser	Ser	Lys	Asn	Pro	Leu	Asn	Asp	Val	Val	Ser	Phe	Lys
50						55					60				
Lys	Ser	Ala	Asp	Asn	Thr	Leu	Asp	Ser	Tyr	Phe	Asp	Asp	Glu	Asp	Lys
65					70					75					80
Pro	Arg	Glu	Glu	Cys	Gly	Val	Val	Gly	Ile	Tyr	Gly	Asp	Ser	Glu	Ala
				85					90					95	
Ser	Arg	Leu	Cys	Tyr	Leu	Ala	Leu	His	Ala	Leu	Leu	His	Arg	Gly	Gln
			100					105					110		
Glu	Gly	Ala	Gly	Ile	Val	Ala	Val	Asn	Asp	Asp	Val	Leu	Lys	Ser	Ile
		115					120					125			
Thr	Gly	Val	Gly	Leu	Val	Ser	Asp	Val	Phe	Asn	Glu	Ser	Lys	Leu	Asp
	130					135					140				
Gln	Leu	Pro	Gly	Asp	Met	Ala	Ile	Gly	His	Val	Trp	Tyr	Ser	Thr	Ala
145					150					155					160
Gly	Ser	Ser	Met	Leu	Lys	Asn	Val	Gln	Pro	Phe	Val	Ala	Asn	Tyr	Lys
			165					170						175	
Phe	Gly	Ser	Val	Gly	Val	Ala	His	Asn	Gly	Asn	Leu	Val	Asn	Tyr	Lys
			180					185						190	
Leu	Leu	Arg	Gly	Glu	Leu	Glu	Glu	Asn	Gly	Ser	Ile	Phe	Asn	Thr	Ser
		195					200					205			
Ser	Asp	Thr	Glu	Val	Val	Leu	His	Leu	Ile	Ala	Ile	Ser	Lys	Ala	Arg
	210					215					220				
Pro	Phe	Leu	Leu	Arg	Ile	Val	Glu	Ala	Cys	Glu	Lys	Ile	Glu	Gly	Ala
225					230				235					240	
Tyr	Ser	Met	Val	Phe	Val	Thr	Glu	Asp	Lys	Leu	Val	Ala	Val	Arg	Asp
			245					250						255	
Pro	His	Gly	Phe	Arg	Pro	Leu	Val	Met	Gly	Arg	Arg	Ser	Asn	Gly	Ala
		260						265					270		
Val	Val	Phe	Ala	Ser	Glu	Thr	Cys	Ala	Leu	Asp	Leu	Ile	Glu	Ala	Thr
		275					280					285			
Tyr	Glu	Arg	Glu	Val	Asn	Pro	Gly	Glu	Val	Val	Val	Val	Asp	Lys	Asp
	290					295						300			

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Gly Val His Ser Ile Tyr Leu Met Pro His Pro Glu His Lys Ser Cys
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Ile Phe Glu His Ile Tyr Phe Ala Leu Pro Asn Ser Val Val Phe Gly
 325 330 335

Arg Ser Val Tyr Glu Ser Arg Arg Ala Phe Gly Glu Ile Leu Ala Thr
 340 345 350

Glu Ala Pro Val Glu Cys Asp Val Gly Ile Ala Val Pro Asp Ser Gly
 355 360 365

Ile Val Ala Ala Leu Gly Tyr Ala Ala Lys Ala Gly Val Pro Phe Gln
 370 375 380

Gln Gly Leu Ile Arg Ser His Tyr Val Gly Arg Thr Phe Ile Glu Pro
 385 390 395 400

Ser Gln Lys Ile Arg Asp Phe Gly Val Lys Leu Lys Leu Ser Pro Val
 405 410 415

Arg Ala Leu Leu Glu Gly Lys Arg Val Val Val Val Asp Asp Ser Ile
 420 425 430

Val Arg Gly Thr Thr Ser Ser Lys Ile Val Arg Leu Leu Lys Glu Ala
 435 440 445

Gly Ala Lys Glu Val His Met Arg Ile Ala Ser Pro Pro Ile Ile Ala
 450 455 460

Ser Cys Tyr Tyr Gly Val Asp Thr Pro Ser Ser Asp Glu Leu Ile Ser
 465 470 475 480

Asn Arg Met Ser Val Glu Glu Ile Lys Glu Phe Ile Gly Ser Asp Ser
 485 490 495

Leu Ala Phe Leu Pro Met Asp Ser Leu Asn Lys Leu Leu Gly Asn Asp
 500 505 510

Ser Lys Ser Phe Cys Tyr Ala Cys Phe Ser Gly Asn Tyr Pro Val Glu
 515 520 525

Pro Thr Gly Lys Val Lys Arg Ile Gly Asp Phe Met Asp Asp Gly Leu
 530 535 540

Ser Gly Asp Met Asp Ser Ile Asp Gly Gly Trp Leu Pro Gly Ser Ser
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7

Arg Val Gln Lys Thr Ile Leu Asn Glu Val Arg Thr Gly
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<212> DNA

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 Ala Ala Ala Thr Asn Lys Tyr Pro Leu Ser Gln Pro Leu Asp Lys Pro
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ttt tgc tcc cta tct caa aag ctc tta tct tta tcc cct aaa acc cat 147
 Phe Cys Ser Leu Ser Gln Lys Leu Leu Ser Leu Ser Pro Lys Thr His
 30 35 40

cct aaa ccc tac aga act ctc atc acc gcc tct tcc aaa aac ccc tta 195
 Pro Lys Pro Tyr Arg Thr Leu Ile Thr Ala Ser Ser Lys Asn Pro Leu
 45 50 55

aac gac gtc att tcg ttt aag aaa tca gct gac aat acc ttg gac tcc 243
 Asn Asp Val Ile Ser Phe Lys Lys Ser Ala Asp Asn Thr Leu Asp Ser
 60 65 70

tat ttt gac gat gac gat aaa ccc cgt gaa gag tgc ggc gtt gtg ggc 291
 Tyr Phe Asp Asp Asp Asp Lys Pro Arg Glu Glu Cys Gly Val Val Gly
 75 80 85

atc tat ggc gac tca gaa gct tca cgc ctt tgc tat tta gca ctt cac 339
 Ile Tyr Gly Asp Ser Glu Ala Ser Arg Leu Cys Tyr Leu Ala Leu His
 90 95 100 105

gcg ctt caa cac cgt ggc caa gaa ggc gcc ggc att gtc gcc gtt aac 387
 Ala Leu Gln His Arg Gly Gln Glu Gly Ala Gly Ile Val Ala Val Asn
 110 115 120

gac gac gtt ctt aag tca att aca ggt gtt ggg tta gta tcc gac gtg 435
 Asp Asp Val Leu Lys Ser Ile Thr Gly Val Gly Leu Val Ser Asp Val
 125 130 135

ttc aat gag tca aag ctt gac caa ctc cct ggt gac atg gca att ggc	483
Phe Asn Glu Ser Lys Leu Asp Gln Leu Pro Gly Asp Met Ala Ile Gly	
140 145 150	
cac gta agg tac tct act gct ggc tct tct atg tta aaa aat gtt cag	531
His Val Arg Tyr Ser Thr Ala Gly Ser Ser Met Leu Lys Asn Val Gln	
155 160 165	
cct ttt gtt gct agt tat aaa ttt ggg tca gtt ggt gtt gcc cat aat	579
Pro Phe Val Ala Ser Tyr Lys Phe Gly Ser Val Gly Val Ala His Asn	
170 175 180 185	
ggt aat tta gtg aat tat aag tta ctg cgt agt gaa cta gag gaa aat	627
Gly Asn Leu Val Asn Tyr Lys Leu Leu Arg Ser Glu Leu Glu Glu Asn	
190 195 200	
ggg tca att ttt aat aca agt tct gat act gag gtt gta ctt cac ctt	675
Gly Ser Ile Phe Asn Thr Ser Ser Asp Thr Glu Val Val Leu His Leu	
205 210 215	
att gct ata tct aaa gct agg cca ttt tta ttg agg att gtt gag gct	723
Ile Ala Ile Ser Lys Ala Arg Pro Phe Leu Leu Arg Ile Val Glu Ala	
220 225 230	
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Cys Glu Lys Ile Glu Gly Ala Tyr Ser Met Val Phe Val Thr Glu Asp	
235 240 245	
aag ttg gtt gcc gta agg gat cct cat ggg ttt agg cca ttg gtt atg	819
Lys Leu Val Ala Val Arg Asp Pro His Gly Phe Arg Pro Leu Val Met	
250 255 260 265	
ggt agg aga agt aat ggt gct gtt gtt ttc gcg tct gag acg tgt gct	867
Gly Arg Arg Ser Asn Gly Ala Val Val Phe Ala Ser Glu Thr Cys Ala	
270 275 280	
ttg gat ttg att gag gct act tat gag agg gag gtg aat cct ggt gag	915
Leu Asp Leu Ile Glu Ala Thr Tyr Glu Arg Glu Val Asn Pro Gly Glu	
285 290 295	
gtt gtt gtt gtg gat aaa gat ggg gtt cag tct att tgt ttg atg cct	963
Val Val Val Val Asp Lys Asp Gly Val Gln Ser Ile Cys Leu Met Pro	
300 305 310	
cat cct gag cgt aaa tct tgt atc ttt gag cat att tac ttt gct ctg	1011
His Pro Glu Arg Lys Ser Cys Ile Phe Glu His Ile Tyr Phe Ala Leu	
315 320 325	

9

cct aat tcg gtc gtg ttt ggg agg tct gtg tac gag tct agg cgt gct	1059
Pro Asn Ser Val Val Phe Gly Arg Ser Val Tyr Glu Ser Arg Arg Ala	
330 335 340 345	
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Phe Gly Glu Ile Leu Ala Thr Glu Ala Pro Val Glu Cys Asp Val Val	
350 355 360	
ata gca gtt cct gac tcg ggt gtc gtg gct gcg ctc ggt tat gct gct	1155
Ile Ala Val Pro Asp Ser Gly Val Val Ala Ala Leu Gly Tyr Ala Ala	
365 370 375	
aaa gca ggg gta ccg ttt caa caa ggt ttg att agg tcg cat tat gtt	1203
Lys Ala Gly Val Pro Phe Gln Gln Gly Leu Ile Arg Ser His Tyr Val	
380 385 390	
ggg agg acg ttc atc gag cca tcg cag aag ata agg gat ttc ggg gtg	1251
Gly Arg Thr Phe Ile Glu Pro Ser Gln Lys Ile Arg Asp Phe Gly Val	
395 400 405	
aag ctt aag ctg tcg ccg gtt agg gcg gtg ttg gag gga aaa aga gtt	1299
Lys Leu Lys Leu Ser Pro Val Arg Ala Val Leu Glu Gly Lys Arg Val	
410 415 420 425	
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Val Val Val Asp Asp Ser Ile Val Arg Gly Thr Thr Ser Ser Lys Ile	
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Val Arg Leu Leu Lys Glu Ala Gly Ala Lys Glu Val His Met Arg Ile	
445 450 455	
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Ala Ser Pro Pro Ile Ile Ala Ser Cys Tyr Tyr Gly Val Asp Thr Pro	
460 465 470	
agt tca gat gag ttg ata tca aat agg atg agt gtg gag gag att aag	1491
Ser Ser Asp Glu Leu Ile Ser Asn Arg Met Ser Val Glu Glu Ile Lys	
475 480 485	
gag ttc att gga tcg gat tcg ctt gct ttt ctg cca atg gat agc ttg	1539
Glu Phe Ile Gly Ser Asp Ser Leu Ala Phe Leu Pro Met Asp Ser Leu	
490 495 500 505	
aat aag ctc tta ggc aat gat tct aaa agc ttt tgc tat gct tgc ttt	1587
Asn Lys Leu Leu Gly Asn Asp Ser Lys Ser Phe Cys Tyr Ala Cys Phe	
510 515 520	

10

tcg ggc aat tac cca gtc gag ccg acg ggt aag gtt aaa agg ata ggg 1635
 Ser Gly Asn Tyr Pro Val Glu Pro Thr Gly Lys Val Lys Arg Ile Gly
 525 530 535

gat ttc atg gat gat gga tta agt gga gat atg gat tcc att gat ggt 1683
 Asp Phe Met Asp Asp Gly Leu Ser Gly Asp Met Asp Ser Ile Asp Gly
 540 545 550

gga tgg cta cca gga agt agt agg gtt caa aag act atc ttg aat gaa 1731
 Gly Trp Leu Pro Gly Ser Ser Arg Val Gln Lys Thr Ile Leu Asn Glu
 555 560 565

gtt aga acc agc taaactttct tttccatggt tgcttttagtt tttgctttgg 1783
 Val Arg Thr Ser
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<213> Nicotiana tabacum

<400> 4

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Pro Leu Ser Gln Pro Leu Asp Lys Pro Phe Cys Ser Leu Ser Gln Lys
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Leu Leu Ser Leu Ser Pro Lys Thr His Pro Lys Pro Tyr Arg Thr Leu
 35 40 45

Ile Thr Ala Ser Ser Lys Asn Pro Leu Asn Asp Val Ile Ser Phe Lys
 50 55 60

Lys Ser Ala Asp Asn Thr Leu Asp Ser Tyr Phe Asp Asp Asp Asp Lys
 65 70 75 80

Pro Arg Glu Glu Cys Gly Val Val Gly Ile Tyr Gly Asp Ser Glu Ala
 85 90 95

Ser Arg Leu Cys Tyr Leu Ala Leu His Ala Leu Gln His Arg Gly Gln
 100 105 110

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Glu Gly Ala Gly Ile Val Ala Val Asn Asp Asp Val Leu Lys Ser Ile
 115 120 125

Thr Gly Val Gly Leu Val Ser Asp Val Phe Asn Glu Ser Lys Leu Asp
 130 135 140

Gln Leu Pro Gly Asp Met Ala Ile Gly His Val Arg Tyr Ser Thr Ala
 145 150 155 160

Gly Ser Ser Met Leu Lys Asn Val Gln Pro Phe Val Ala Ser Tyr Lys
 165 170 175

Phe Gly Ser Val Gly Val Ala His Asn Gly Asn Leu Val Asn Tyr Lys
 180 185 190

Leu Leu Arg Ser Glu Leu Glu Glu Asn Gly Ser Ile Phe Asn Thr Ser
 195 200 205

Ser Asp Thr Glu Val Val Leu His Leu Ile Ala Ile Ser Lys Ala Arg
 210 215 220

Pro Phe Leu Leu Arg Ile Val Glu Ala Cys Glu Lys Ile Glu Gly Ala
 225 230 235 240

Tyr Ser Met Val Phe Val Thr Glu Asp Lys Leu Val Ala Val Arg Asp
 245 250 255

Pro His Gly Phe Arg Pro Leu Val Met Gly Arg Arg Ser Asn Gly Ala
 260 265 270

Val Val Phe Ala Ser Glu Thr Cys Ala Leu Asp Leu Ile Glu Ala Thr
 275 280 285

Tyr Glu Arg Glu Val Asn Pro Gly Glu Val Val Val Val Asp Lys Asp
 290 295 300

Gly Val Gln Ser Ile Cys Leu Met Pro His Pro Glu Arg Lys Ser Cys
 305 310 315 320

Ile Phe Glu His Ile Tyr Phe Ala Leu Pro Asn Ser Val Val Phe Gly
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Arg Ser Val Tyr Glu Ser Arg Arg Ala Phe Gly Glu Ile Leu Ala Thr
 340 345 350

Glu Ala Pro Val Glu Cys Asp Val Val Ile Ala Val Pro Asp Ser Gly
 355 360 365

12

Val Val Ala Ala Leu Gly Tyr Ala Ala Lys Ala Gly Val Pro Phe Gln
370 375 380

Gln Gly Leu Ile Arg Ser His Tyr Val Gly Arg Thr Phe Ile Glu Pro
385 390 395 400

Ser Gln Lys Ile Arg Asp Phe Gly Val Lys Leu Lys Leu Ser Pro Val
405 410 415

Arg Ala Val Leu Glu Gly Lys Arg Val Val Val Val Asp Asp Ser Ile
420 425 430

Val Arg Gly Thr Thr Ser Ser Lys Ile Val Arg Leu Leu Lys Glu Ala
435 440 445

Gly Ala Lys Glu Val His Met Arg Ile Ala Ser Pro Pro Ile Ile Ala
450 455 460

Ser Cys Tyr Tyr Gly Val Asp Thr Pro Ser Ser Asp Glu Leu Ile Ser
465 470 475 480

Asn Arg Met Ser Val Glu Glu Ile Lys Glu Phe Ile Gly Ser Asp Ser
485 490 495

Leu Ala Phe Leu Pro Met Asp Ser Leu Asn Lys Leu Leu Gly Asn Asp
500 505 510

Ser Lys Ser Phe Cys Tyr Ala Cys Phe Ser Gly Asn Tyr Pro Val Glu
515 520 525

Pro Thr Gly Lys Val Lys Arg Ile Gly Asp Phe Met Asp Asp Gly Leu
530 535 540

Ser Gly Asp Met Asp Ser Ile Asp Gly Gly Trp Leu Pro Gly Ser Ser
545 550 555 560

Arg Val Gln Lys Thr Ile Leu Asn Glu Val Arg Thr Ser
565 570